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14. ABSTRACT
Plecktrin Homology (PH) domains are commonly thought of as membrane-targeting modules involved in signaling pathways that bind phosphoinositides (PPIns) with high affinity and specificity. In a recent study of S. cerevisiae, however, the vast majority demonstrated little affinity or specificity for PPIns (Yu et al, 2004). I show comparable results for selected human PH domains, with one that is high affinity and PPIns-specific, while the remainder are low to moderate affinity and promiscuous for PPIns. I outline two instances where protein-protein and protein-phosphoinositide interactions may account for specific membrane targeting observed in vivo. First, SH3BP-2 PH was identifed as highly specific for the membrane lipid PtdIns(3,4)P2, and targets the host protein to the membrane. Second, FAPP1- and OSBP PH domains possess comparable affinities for Golgi- and plama membrane (PM)-enriched PPIns in vitro, although they both localize to the Golgi (not the PM) in vivo, possibly by directly interacting with the Golgi GTPase Arf1. In vitro binding studies suggest that delocalized electrostatic attraction between the basic protein and acidic phospholipids play a prominent role in these interactions. Additionally, I have solved the crystal structure of a related member of this PH domain family in complex with PPIns.

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Introduction

The primary goal of my studies has been to understand how PH domain recognition of phospholipids and of other proteins contributes to signaling by the wide array of molecules that contain PH domains – the 11th most common domain in the human genome, and one that is present in many proteins implicated in breast cancer (Cesareni *et al*, 2004; Schmidt and Hall, 2002). Elucidation of the nature of these PH domain interactions (and how they are regulated) in sufficient detail may suggest approaches for inhibiting these interactions pharmacologically. This report will enumerate the advances that I have made towards reaching these goals. In addition to the proposed *in vitro* phospholipid binding studies and *in vivo* cellular localization studies, I have also characterized a specific PH domain-alkylphospholipid drug interaction *in vitro*, and have completed the structural determination of a member of a poorly characterized class of PH domains bound to phospholipid. Members of this class include the mammalian PH domains of oxysterol-binding protein-1 (OSBP1) and four-phosphoinositide adaptor protein-1 (FAPP1), whose host proteins are involved in coordinating budding and fission events at the Golgi for the generation of cargo transporters targeted for fusion with the plasma membrane (Itoh & De Camilli, 2004; Roth 2004). At least two additional members of this class of PH domains (FAPP2 and OSBP2) have been associated with breast cancer development or progression (Fournier *et al*, 1999; Scanlan *et al*, 2001).

Following the format of my proposed Statement of Work, my ultimate accomplishments are summarized as follows:

Task 1: Investigate affinity and specificity of phosphoinositide binding to isolated human PH domains

I have analyzed the phosphoinositide binding specificity of 21 of the 66 phylogenetic representative human PH domains proposed for study with lipid overlay assays (dot blot) and SPR lipid-binding assays, as previously described (Kavran *et al*, 1998; Yu *et al*, 2004). An extensive literature search confirmed that another 8 PH domains that have already been characterized for phosphoinositide binding *in vitro* (Yu *et a*l, 2006; Rajala *et a*l, 2005; Yan *et al*, 2005; Skowronek *et al*, 2004; Saxena *et al*, 2002; Dowler *et al*, 2000; Fleming *et al*, 2000). As expected from our prior analysis of yeast PH domains, the majority of the human PH domains selected bind phosphoinositides promiscuously and with only low affinity (**Table 1**). Six of the human PH domains tested (and only one yeast PH domain) possess high affinity and specificity for phosphoinositides (either for PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, or PtdIns(3,4)P_2/PtdIns(3,4,5)P_3; see **Figure 1**),. The two human PH domains of OSBP1 and FAPP1 represent a separate class of PH domains that will be discussed in more detail below. The corresponding yeast orthologs of mammalian OSBP are Osh1p and Osh2p (Lehto *et al*, 2001; Yu *et al*, 2004).

The OSBP/FAPP/Osh PH domain family has been at times both poorly characterized and mischaracterized both *in vivo* and *in vitro*. Several were initially identified as PtdIns4*P*-specific *in vitro*, and, since that time, have been consistently misused as markers of cellular PtdIns4*P in vivo* (Várnai and Balla, 1998; Dowler *et al*, 2000). In contrast to reported PtdIns4*P* specificity, I have determined that the PH domains of OSBP1 and FAPP1 display *in vitro* binding affinities

that are comparable between PtdIns4*P*- (relative K_d 3.5 and 21 μM, respectively) and PtdIns(4,5)P_2-containing surfaces (relative K_d 3.3 and 17 μM, respectively), as measured by SPR lipid-binding assays (**Figure 2**). These results are consistent with those reported for the fusion PH domain constructs in an earlier study (Levine & Munro, 2002). When the assay was expanded to include other phosphoinositides (clear area of **Table 2**), OSBP1 was found to have comparable *in vitro* binding affinities for all phosphoinositides tested (relative K_d 3.2-3.8 μM), except for the two-fold weaker affinity of PtdIns3*P* surfaces (relative K_d 6.5 μM) and no apparent binding for PtdIns5*P* surfaces. These data mirror published *in vitro* binding affinities of the yeast ortholog Osh1p PH, and sharply contrast with the high affinity and PtdIns(4,5)P_2 specificity of PLCδ PH (shaded area of **Table 2**). Interestingly, FAPP1 PH appears to have an intermediate specificity for phosphoinositides; in addition to the comparable binding affinities for PtdIns4*P*- and PtdIns(4,5)P_2-containing surfaces (relative K_d 21 and 17 μM, respectively), it has a two-fold weaker affinity for PtdIns(3,5)P_2-containing surfaces (relative K_d 31.4 μM), but displays no binding for other phosphoinositides. In some instances, dimeric GST-tagged FAPP1 PH demonstrated more substantial binding to phosphoinositide-containing surfaces, indicating that the relative binding affinity of monomeric FAPP1 PH is likely to be 2-5-fold weaker (e.g.- for PtdIns(3,4)P_2-containing surfaces, on the order of ~65-130 μM (data not shown)). The reasons for such discrepancies in relative binding data between these two highly homologous PH domains remains unclear at the present time.

Another outcome of these studies was the identification of a human PH domain with unexpected phosphoinositide-binding properties. The PH domain of SH3BP-2 (3BP-2) is highly specific for PtdIns(3,4)P_2-containing surfaces, although with a lower estimated relative binding affinity than other 3-phosphoinositide binding PH domains like Akt1/PKBα PH (**Figure 3**). The K_d is 0.3-0.5 μM for the dimeric GST-PH fusion construct (and an estimated ten-fold weaker for monomer (Kavran *et al*, 1998)). SH3BP-2 is an adaptor protein with a versatile role in a variety of cell types. It is in the same phylogenetic PH domain class as that of PtdIns(3,4)P_2/PtdIns(3,4,5)P_3-specific DAPP1 PH (unpublished results), and, like DAPP1 PH, is translocated to the plasma membrane upon PI3K activation (http://www.signaling-gateway.org/). These results are consistent with the protein's PI3K-dependent roles as a positive regulator of IL-2 gene induction in T cells (Deckert *et al*, 1998), NK cell-mediated cytotoxicity (Jevremovic *et al*, 2001), and FcεRI-induced degranulation and signal amplification in mast cells (Sada *et al*, 2002).

Task 2: Determine the subcellular localization of the PH domains

The Alliance for Cell Signaling (AfCS) laboratory at Stanford has provided *in vivo* mouse PH domain localization data for over two-thirds of the 66 human PH domains classes listed in our original proposal (http://www.signaling-gateway.org/). The strong homology of human and mouse PH domain sequences obviated the need to repeat such a large scale *in vivo* cellular localization study with human PH domains. The overall distribution of cytosolic versus membrane- or puncta-localized PH domains is comparable to that of our prior yeast PH domain study (Yu *et al*, 2004). Rather, a handful of potentially useful PH domains selected from the AfCS study that localize to cellular membranes *in vivo* could be analyzed for their *in vitro* binding affinities at a later date.

In order to determine the cellular localization of OSBP1 PH and FAPP1 PH *in vivo*, I transfected GFP fusion constructs into live NIH-3T3 and HeLa cell lines. The fluorescence localization in these cells was punctate in nature (**Figure 4**), consistent with Golgi membrane localization, as found in previous studies (Levine & Munro, 2002; Godi *et al*, 2004; Balla *et al*, 2005). The *in vivo* data is also consistent with the reported roles of this class of PH domains in coordinating budding and fission events at the Golgi for the generation of cargo transporters targeted for fusion with the plasma membrane (Itoh & De Camilli, 2004; Roth 2004). The data are seemingly at odds, however, with the aforementioned *in vitro* data demonstrating the promiscuity (and relatively weak affinity, in the case of FAPP1 PH) for phosphoinositide-containing surfaces. Specifically, the observation that OSBP/FAPP/Osh1 PH family members target to the PtdIns4*P*–rich Golgi rather than the PtdIns(4,5)P_2-rich plasma membrane *in vivo*, despite no apparent difference in *in vitro* binding affinities for their respective phosphoinositide, is unexpected. The reconciliation of *in vitro* data demonstrating promiscuity in phosphoinositide binding, and *in vivo* Golgi localization of OSBP PH and FAPP1 PH is expected to involve a non-phosphoinositide component (Levine & Munro, 2002), and is weakly supported by previous *in vitro* pulldown studies preformed here (*Task 3*) and elsewhere (Godi *et al*, 2004).

Task 3 Putative interacting proteins of PH domains

In my attempt to understand the role of moderate affinity and promiscuous PH domains, I have attempted to reconcile at least two experimental observations of the OSBP/ FAPP family that remain unresolved. The first - and the subject of this task - addresses the discrepancy between *in vitro* data (promiscuity in phosphoinositide binding) and *in vivo* data (Golgi localization). More specifically, how are these PH domains correctly targeted to the Golgi *in vivo* in the absence of major differences in their *in vitro* affinity and selectivity for (Golgi-enriched) PI(4)P versus (plasma membrane-enriched and more numerous) PtdIns(4,5)P_2? This question is *apropos* for all PH domains that bind phosphoinositides with low affinity and promiscuity that are nevertheless targeted to membranes and/or puncta. The second discrepancy will be discussed in the next section (*Task 4*).

Since phosphoinositide binding alone cannot account for the specific subcellular localization of several PH domains – particularly in the case of the OSBP/FAPP/Osh1 PH family – it has been suggested that other targets, particularly proteins, may help define their localization. Levine and Munro observed that Golgi targeting of the OSBP1 PH domain requires both PtdIns4*P* and a second PI4K-independent determinant, which they suggested from genetic studies might be Arf1, a Golgi small GTPase (Levine & Munro, 2002). More recently, the PH domains of both OSBP1 and FAPP1 were found to specifically and directly interact with Arf1 *in vitro* (Godi *et al*, 2004). I have utilized purified myristoylated Arf1 (DNA construct provided by Paul Randazzo (Manser & Leung, 2002)) to test its reported interaction with GST-tagged FAPP1 PH and OSBP PH using *in vitro* pulldown assays. Results confirm what appears to be a relatively weak and non-robust interaction *in vitro* (**Figure 5**). Although the PH domain literature is replete with instances of *in vitro* pulldowns of putative protein partners, there are inherent limitations in relying on this approach to prove direct, biologically significant PH domain-protein interactions *in vivo*.

There appears to be some consensus that both Arf1 and PtdIns4*P* are both necessary to provide a sufficiently strong interaction with the PH domains *in vivo* (Itoh & De Camilli, 2004; Godi *et al*, 2004; Levine & Munro, 2002). The challenge is to present both the myristoylated Arf1 and PtdIns4*P* in sufficient proximity to one another to allow simultaneous interactions with the PH domains, and to maintain a cellular membrane-like structure that would convincingly demonstrate the likelihood of such an interaction occurring *in vivo*. One possible methodology would be to generate combined myrArf1/phosphoinositide (PtdIns4*P*:PC) vesicles (similar to Randazzo 1997) and quantitatively measure the binding affinity of the interaction using SPR binding assays.

Task 4: Biochemical and Structure Determination of selected PH domains

In Vitro Biochemical Characterization of OSBP/Osh1p PH

One of the primary motivations of this study is to structurally characterize PH domain-ligand (protein or phosphoinositide) interactions in detail. While a possible soluion to the discrepancy between *in vitro* binding data and *in vivo* cellular localization was addressed in *Task 3*, a basic question still remains unanswered concerning the similar *in vitro* phosphoinositide binding affinities. All previously published PH domain structures (liganded or liganded) interact with two adjascent ligand determinants (whether the 3-*P* and the 4-*P* of DAPP1 PH/Ins(1,3,4,5)P4 complex, the 4-*P* and the 5-*P* of PLCδ PH/Ins(1,4,5)P3 complex, or simply two free phosphate groups of unliganded DAPP1 PH (DiNitto *et al*, 2003)). As phosphate groups on the inositol head group are the major PH domain determinants, how (and why) would a PH domain recognize a monophosphoinositide and diphosphoinositide with similar binding affinities? What are the structural determinants that account for PH domain promiscuity? I am addressing this question using several approaches.

Upon performing a gel filtration assay, I observed that while some [^{3}H]-Ins(1,4,5)P_3 (PtdIns(4,5)P_2 headgroup) coelutes with GST-PLCδ PH, no [^{3}H]-Ins(1,4,5)P_3 is detected coeluting with the GST-PH domains of Osh1p, OSBP, or FAPP1 (data not shown), suggesting that the Osh1p/OSBP/FAPP1 PH domain family bind to Ins(1,4,5)P_3 weakly (<1 μM). To identify which phosphoinositide determinants (i.e. inositol phosphates ring moieties, the glycerol moiety or nearby acyl chains) are involved in PH domain binding, I performed SPR lipid competition studies, preincubating inositol headgroups/phosphoinositide constructs of different lengths with the PH domains of OSBP and Osh1p before applying them to phosphatidylinositol-containing surfaces (3% PtdIns4*P* or PtdIns(4,5)P_2 in a PC background) (**Figure 6**). The results were consistent with gel filtration data: **1**) Ins(1,4,5)P_3 does not compete OSBP/Osh1p PH off either PtdIns4*P*- or PtdIns(4,5)P_2-containing surfaces, suggesting headgroup contacts alone are insufficient to account for entire phosphoinositide surface binding *in vitro*; **2**) short-chain phosphoinositides diC4-PtdIns4*P* and diC4-PtdIns(4,5)P_2 compete OSBP/Osh1p PH off PtdIns4*P*- and PtdIns(4,5)P_2–containing surfaces only modestly at best, suggesting that the glycerol moiety and acyl side chains are insufficient to account for entire phosphoinositide surface binding *in vitro*; and **3**) both highly negatively charged InsP_6 and Ins(1,3,4,5,6)P_5 effectively compete OSBP/Osh1p PH off PtdIns4*P*- and PtdIns(4,5)P_2–containing surfaces, suggesting the effecttiveness of electrostatic interactions. By contrast, all inositol phosphates and phosphoinositides tested (except diC4-PtdIns4*P*) compete PLCδ PH off PtdIns(4,5)P_2–

containing surfaces as expected, since PLCδ PH, like many well-characterized high affity PH domains, strongly interacts with phosphoinositides headgroup (Lemmon *et al*, 1995).

To investigate whether delocalized electrostatic attraction rather than phosphoinositide-specific interactions accound for the relatively strong binding of this PH domain family to phosphoinositide-containg surfaces *in vitro*, I tested Osh1p PH domain binding to phosphatidylserine-containing surfaces (20% PtdSer in a PC background) (**Figure 7A**). Osh1p PH, unlike PLCδ PH, bound robustly to PtdSer-containing surfaces *in vitro*, with relative binding affinites on the order of that observed for PPIns surface binding (K_d of 2.2 μM versus 2.8-3.5 μM, respectively). Osh1p PH domain binding to mixed PtdSer/PtdIns surfaces (3% PPIns/ 20% PtdSer in a PC background) *in vitro* are also comparable (data not shown). Additionally, SPR lipid competition studies demonstrate that inositol headgroups/ phosphoinositide constructs compete Osh1p PH off the PtdSer-containing surfaces largely based on the extent of phosphate substitution on the inositol ring of the competitor (**Figure 7B**). All these results are consistent with the predominance of delocalized electrostatic attraction in OSBP/FAPP1/Osh1p PH domain family binding to phospholipid surfaces *in vitro*. Therefore, this is a unique class of PH domains from any published to date.

Structural Determination of Osh1p PH

To identify the specific contribution of the ligand determinants to PH domain binding, I proposed to determine the structure of the PH domain complexed with phosphoinositides. I have prepared crystals of monomeric His-tagged Osh1 PH complexed with the soluble, short chain (C4) derivatives of both PtdIns4*P* and PtdIns(4,5)P_2 (**Figure 8A**). I have collected a full structure data set of the SO_4– and PtdIns4*P*–complexed Osh1 crystals at high resolution, and have identified a molecular replacement solution working with DAPP1 PH (see **Table 3** for details). Both Osh1p PH structures exhibit a standard PH domain fold (Ferguson *et al* 2000; Lietzke *et al* 2000), with seven β-strands forming two orthogonal anti-parallel β-sheets and capped by a C-terminal α-helix, as well as three loops (β1/β2 (VL1), β3/β4 (VL2), and β6/β7 (VL3)) that define the lipid binding pocket, possessing conserved, mainly basic residues characteristic of high affinity phosphoinositide binding (**Figure 8B**). While there is considerable overlap of the Cα backbone between the SO_4-complexed and the PtdIns4*P*-liganded Osh1p PH domain, two regions show a ~0.5 to 2 Å displacement between the two structures: the β3/β4 loop and a Gly residue in the β6/β7 loop (Gly-350) (**Figure 9**). This is only the second ligand-induced conformation of PH domain structures observed to date. Upon Ins(1,3,4,5)P_4 binding to Akt1/PKBα PH, both the β3/β4 loop and the β6/β7 loop are displaced up to 7.4-7.6 Å (Milburn *et al* 2003; Auguin *et al* 2004). In both Akt1/PKBα PH and Osh1p PH, the majority of the β3/β4 loop is not well-defined in the unliganded or SO_4-complexed structure, but is well-defined in the corresponding phosphoinositide-liganded structure. Moreover, in both instances, a short, acidic α-helix or helical-like structure are formed within the β3/β4 loop of the phosphoinositide-liganded structure. (In Akt1/PKBα PH, the sequence is $_{44}$DVDQRE$_{49}$, and in Osh1p PH, the sequence is $_{316}$DQAD$_{319}$ (Milburn et al 2003)).

Similar to most PH domains, the Osh1p PH has a polarized electrostatic potential, with an extensive positively-charged surface around the phosphoinositide-binding pocket, and a smaller negatively-charged surface, which may interact with putative protein binding partners such as Gβγ (Lodowski *et al* 2003) or small GTPases like Arf1, Rho1, or Cdc42 (Levine and Munro

2002; Godi *et al* 2004; Kozminski *et al* 2006). The electrostatic surface model does demonstrate a small, negatively-charged cleft that may be a putative Arf1-interacting region (**Figure 8C**). Even more promising, the electrostatic surface model reveals a uniquely hydrophobic region around the β1/β2 loop, possessing a cluster of hydrophobic residues (i.e. Phe-294, Trp-291, Tyr-298) that are well-positioned to potentially insert into Golgi membranes *in vivo*, after residues with basic side chains neutralize the negatively-charged membrane phospholipids.

While the SO_4 ligand density in the binding site is easily identified, the C4-PtdIns4*P* ligand density is not as easily defined (**Figure 10A, B**). The density of the 4-phosphate and at least one of the hydroxyl groups on the inosiol ring are relatively clear, but the remaining structure, including the the 1-phosphate and the area surrunding it, as well as the two acyl chains are smeared. We attribute this smearing to multiple albeit limited conformations of the phosphoinositide in the ligand binding site. Our best structure to date places the phosphoinositide ligand in a less than ideal conformation, although the phosphoinositide-interacting residues in this structure are conserved among other high affinity PH domains (Tyr-313, Lys-290, Arg-301, Lys-299, and Arg-325), with relatively few OSBP/Osh1p PH-unique sequences (Gln-296 and Thr-292) (**Figure 11A, B**). Additionally, several mutational studies confirm the prominence of these interactions in the OSBP/Osh1p PH domain family (Yu *et al*, 2004; Levine and Munro, 1998, 2001, 2002; Godi *et al* 2004; Kumagai *et al* 2007). Finally, both SO_4 and C4-PtdIns4*P* ligands are positioned in a similar manner as other liganded PH domains whose structures have already been determined (**Figure 12A, B**).

PH domains as drug targets in cancer therapy

Our original view of PH domains predicted that phosphoinositide-binding PH domains would make poor targets for pharmacological intervention, since: 1) most phosphoinositide-recognition events are essentially the same; and, 2) drugs likely to target PH domains are very highly charged, which leads to delivery problems. I have had the opportunity to test the PH domain-binding properties of perifosine, a C_{18}-alkylphospholipid drug that has recently completed phase I trials (Van Ummersen *et al*, 2004; Crul *et al*, 2004) as an anti-cancer agent. Earlier immunoprecipitation studies suggested that perifosine specifically inhibits Ser/Thr phosphorylation and kinase activation of Akt1/PKB *in vivo* and *in vitro* (Kondapaka *et al*, 2003). Myristoylated Akt1/PKB, which is targeted directly to the plasma membrane in a PH domain-independent manner, is unaffected by perifosine treatment. I therefore surmised that perifosine might act by directly interfering with phosphoinositide binding of the PKB PH domain. In my last report, I presented SPR binding data suggesting that perifosine specifically competes with phosphoinositides for binding to the PH domain of Akt1/PKB (EC_{50} 26 μM), while it competes substantially less for binding to the PH domain of PLCδ, and not at all for the PH domains of DAPP1 and FAPP1. These studies indicate that perifosine likely binds directly to the phosphoinositide-binding site of the PKB PH domain. Follow up studies to determine the binding affinity of the Akt1/PKB PH-perifosine interaction using isothermal titration calorimetry (ITC) were difficult to perform due to the relatively high concentrations of PH domain required for the study, but confirmed the relatively low binding affinity (~30 μM) of the interaction (**Figure 13**). Thus, although perifosine interacts specifically with PKB PH *in vitro*, other means appear to be necessary – perhaps other segments of Akt1/PKB or even an additional cofactor – to "boost" the binding affinity of this interaction.

Key Research Accomplishments

- The binding affinities of monomeric PH domains for OSBP, Osh1p, FAPP for PPIns-containing surfaces using SPR assays demonstrates that this family of PH domains is promiscuous and has moderate-to-high affinity for binding to PPIns-containing surfaces
- Short-chain PPIns are only weakly and partially able to compete OSBP/Osh1p PH off PPIns-containing surfaces, indicating that phosphoinositide determinants are insufficient to account for full binding to PPIns-containing surfaces in SPR lipid competition assays.
- Robust OSBP/Osh1p PH binding to PtdSer-containing surfaces and mixed phosholipid surfaces demonstrate that delocalized electrostatic attraction is the key component of PH-PPIns surface interactions.
- Crystal structure of SO_4– and PtdIns4*P*–liganded Osh1p PH provides evidence that most PtdIns4*P*–interacting ressidues are conserved in high affinity, PPIns-specific PH domains, but have a far weaker interaction due in part to the lack of a second phosphate determinant and fewer hydrogen bonding interactions per residue.
- Weak myrArf1 interactions with OSBP PH and FAPP1 PH were demonstrated *in vitro* using GST pulldown assays.
- The alkylphospholipid drug perifosine has a relatively low binding affinity for Akt1/PKBα PH according to ITC studies, despite its rather strong selectivity for the domain.
- Partially characterized novel 3-phosphoinositide-binding SH3BP2 PH domain.

Reportable Outcomes

- Cloned multiple monomeric and fusion tagged PH domain constructs.
- Developed SPR lipid competition assays.
- Developed Western blot for myristoylated Arf1 detection.
- Determined Osh1p PH domain crystal structure.
- Completed *in vitro* binding analysis of Akt1/PKBα PH-perifosine interaction.

Conclusion

Consistent with our previous yeast genome wide study (Yu *et al*, 2004), the vast majority of PH domains demonstrate low affinity, promiscuous binding of phosphoinositides, and relatively few demonstrate high affinity, phosphoinositide-specific binding. A third relatively understudied group of PH domains possess moderate affinity and promiscuity for phosphoinositides, and often target to particular cellular compartments *in vivo*. One group from this class, OSBP/FAPP family, target to the Golgi *in vivo*, and are related to the yeast PH domains of Osh proteins (Lehto *et al*, 2001). Interestingly, at least two members of this group (FAPP2 and OSBP2) have been associated with breast cancer development or progression (Fournier *et al*, 1999; Scanlan *et al*, 2001).

I have continued my *in vitro* analysis of monomeric OSBP PH and FAPP PH by: (1) confirming that their relative binding affinities for Golgi-specific PI(4)P are very similar to that

of plasma membrane-specific $PI(4,5)P_2$; (2) demonstrating that the binding affinity of monomeric OSBP PH for $PI(3,4)P_2$ is comparable to that for PI(4)P and $PI(4,5)P_2$ (indicative of its intrinsic promiscuity for phosphoinositides), whereas the binding affinity of monomeric FAPP1 PH for $PI(3,4)P_2$ is much weaker. The *in vitro* binding affinities of OSBP PH and FAPP1 PH for other mono- and diphosphoinositides will subsequently be measured.

It has been suggested that a non-phosphoinositide cofactor is required to explain the discrepancy that members of the OSBP/FAPP family are targeted to the Golgi *in vivo* in the absence of any difference in the *in vitro* binding affinities for (Golgi-enriched) PI(4)P versus (PM-enriched) $PI(4,5)P_2$ (Godi *et al*, 2004). The Golgi-based GTPase Arf1 has been proposed to be the cofactor based on *in vitro* pulldown studies, results that are consistent with my study. To quantify this interaction, I will purify myristoylated Arf1 and combine it with PI(4)P (in a PC background) to create protein/lipid vesicles. These vesicles should be amenable to binding affinity determination by SPR assays.

A second unresolved question involving the OSBP/FAPP PH family concerns the identification of structural determinants that account for PH domain promiscuity given that: 1) the relative binding affinities for the PH domains for the monophosphoinositide PI(4)P and the diphosphoinositide $PI(4,5)P_2$ are similar; and 2) phosphate groups on the inositol head group are major PH domain determinants. I am attempting to solve the crystal structure of OSBP PH domain alone and complexed with the phosphoinositides PI(4)P and $PI(4,5)P_2$ to address this issue. I have observed multiple crystals in a variety of conditions tested in the unliganded form, and two promising crystals of OSBP PH domain liganded to PI(4)P, which are currently under further development.

Finally, after having demonstrated the specificity of the anticancer drug perifosine for Akt1/PKB PH for the last reporting period, I have currently demonstrated perifosine's low binding affinity for Akt1/PKB PH as measured by ITC. Understanding the structural basis for this PH domain-specific interaction will allow me to elucidate the determinants involved, contributing to the quest for an effective anticancer therapy.

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Table 1A Yeast PH Domains

Num1p		
Cla4p	Boi1p	
Skm1p	**Boi2p**	
Osh1p	***Osh2p***	
Ask10p	Syt1p	Caf120p
Bem2p	Ugt51p	Gcs1p
Cdc24p	Ybl060p	Opy1Ap
Opy1Bp	Yhr131p	Tus1p
Osh3p	**Yil105cp**	Yhr155wp
Sip3p	Ylr187wBp	Ynl144p
Spo14p	**Ynl047cp**	Ypr091cp
Spo71Bp	Ypr115wp	Spo71Ap
Stt4p	Bem3p	Ylr187wAp

B Human PH Domains

PLCδ$_1$		
Tiam1-Ct	DAPP1 / PHISH	**Gab2**
Cytohesin 2 / ARNO	*TAPP1-Ct*	
*FAPP1**	*OSBP1**	*Trio-Nt*
Vav1	AP20 / LL5β	PLD1
Dbl	FGD1-Nt	**Dok1**
Sos1	*FGD1-Ct*	Pleckstrin-Nt
IRS1	**DAGKδ**	Dynamin 1
PEPP1	Syntrophin-3	Grb14
TAPP1-Nt	Tiam1-Nt	Ipl
βARK1	KIAA0053, RhoGAP25	Myosin X-Nt

Table 1A, B Yeast and Human PH domain affinity *in vitro* and localization *in vivo*

Color: High affinity and PtdIns(4,5)P_2-specific in red, High affinity and PtdIns(3,4)P_2/PtdIns(3,4,5)P_3-specific in green, Moderate affinity and promiscuous in purple, Low affinity and promiscuous in blue. Font: Cytosolic and nuclear localization is regular, Plasma membrane localization is **bold**, Punctate localization is *italicized* (see Yu *et al*, 2004 for details).

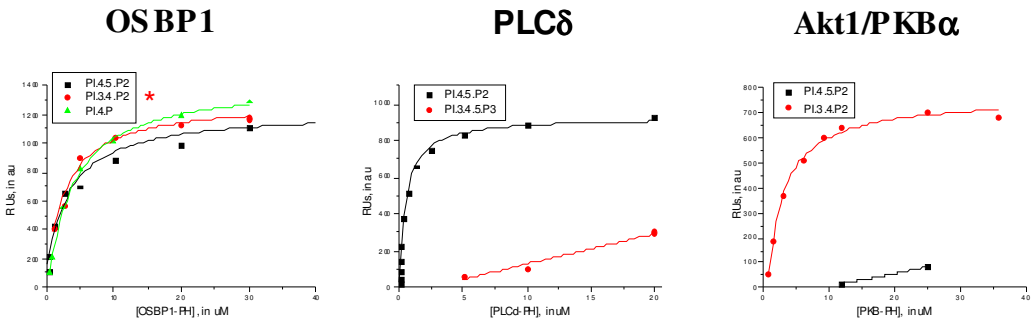
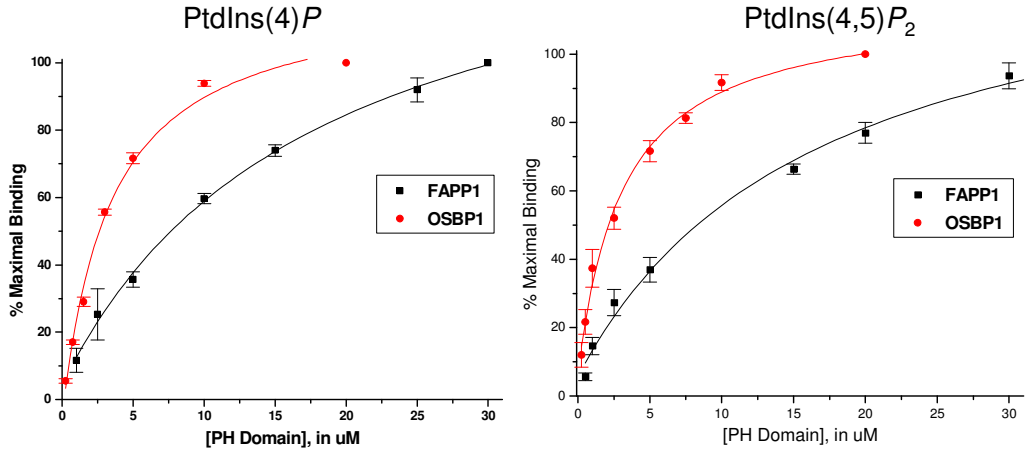


Figure 1: SPR binding data for OSBP PH suggest promiscuity for phosphoinositides.
SPR binding assay protocol detailed in Yu *et al*, 2004. BIAcore response is based on refractive index changes that accompany protein binding to a lipid-coated chip surface. The apparent Kd is calculated from repeated iterations of Y=(Rmax*((1/Kd)*X)/(1+((1/Kd)*X)))+cf, where Rmax is the maximal response and cf is the correction factor.



PH Domain	PtdIns3*P*	PtdIns4*P*	PtdIns5*P*	PtdIns(3,4)*P2*	PtdIns(3,5)*P2*	PtdIns(4,5)*P2*	PtdIns(3,4,5)*P3*
OSBP	6.5 ± 1.3	3.4 ± 0.7	NB	3.8 ± 1.1	3.4 ± 0.4	3.2 ± 0.6	3.6 ± 1.4
FAPP1	NB	17.8 ± 2.4	NB	NB	31.4 ± 5.5	16.7 ± 3.6	NB
PLCd	> 100 µM	131 ± 19	NB	NB	76.0 ± 4.7	0.68 ± 0.28	NB
Osh1	6.2 ± 1.3	2.8 ± 0.8	------	------	3.5 ± 0.8	3.0 ± 1.0	------
Osh2	1.5 ± 0.2	1.3 ± 0.2	------	------	1.0 µM	1.1 ± 0.3	------

Figure 2 SPR binding curves for OSBP PH and FAPP1 PH for PtdIns(4,5)P_2 and PtdIns4*P* surfaces. SPR binding assay protocol detailed in Yu *et al*, 2004. BIAcore response is based on refractive index changes that accompany protein binding to a lipid-coated chip surface. The apparent Kd is calculated from repeated iterations of Y=(Rmax*((1/Kd)*X)/(1+((1/Kd)*X)))+cf, where Rmax is the maximal response and cf is the correction factor.

Table 2 SPR binding data for OSBP PH and FAPP1 PH for all phosphoinositide surfaces
OSBP PH and FAPP1 PH data showed alongsude published data of PLCδ PH, Osh1 PH, and Osh2 PH (Yu *et al*, 2004). Note that no positive controls have been identified for PtdIns5*P*.

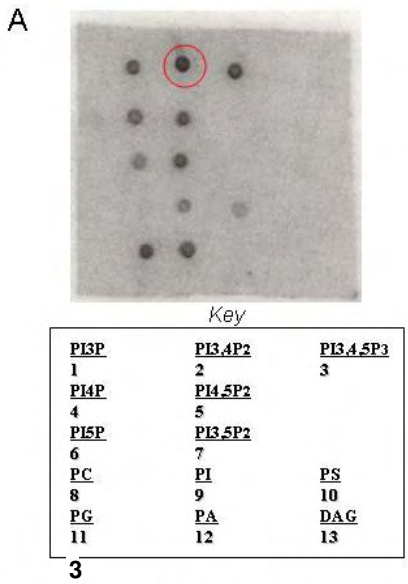


Fig.1A SH3BP-2 PH domain is a moderate affinity, PI(3,4)P_2-specific PH domain.
Protein-Lipid Overlay Assay protocol detailed in Yu *et al*, 2004
The phospholipids are spotted at the positions indicated on the key. The intensity of the spots are indicative of radiolabeled protein bound to lipid. GST-PH domain fusion of SH3BP-2 shows selectivity for PI(3,4)P_2.

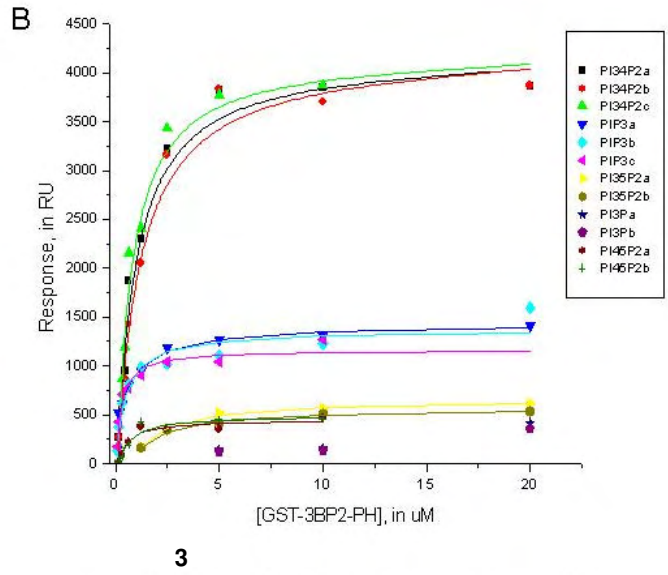


Fig.1B SH3BP-2 PH domain is a moderate affinity, PI(3,4)P_2-specific PH domain
SPR Binding Assay protocol detailed in Yu *et al*, 2004
BIAcore response is based on refractive index changes that accompany protein binding to a lipid-coated chip surface. The apparent Kd is calculated from repeated iterations of: $Y=(Rmax*((1/Kd)*X)/(1+((1/Kd)*X)))+ff$, where Rmax is the maximal response and cf is the correction factor.

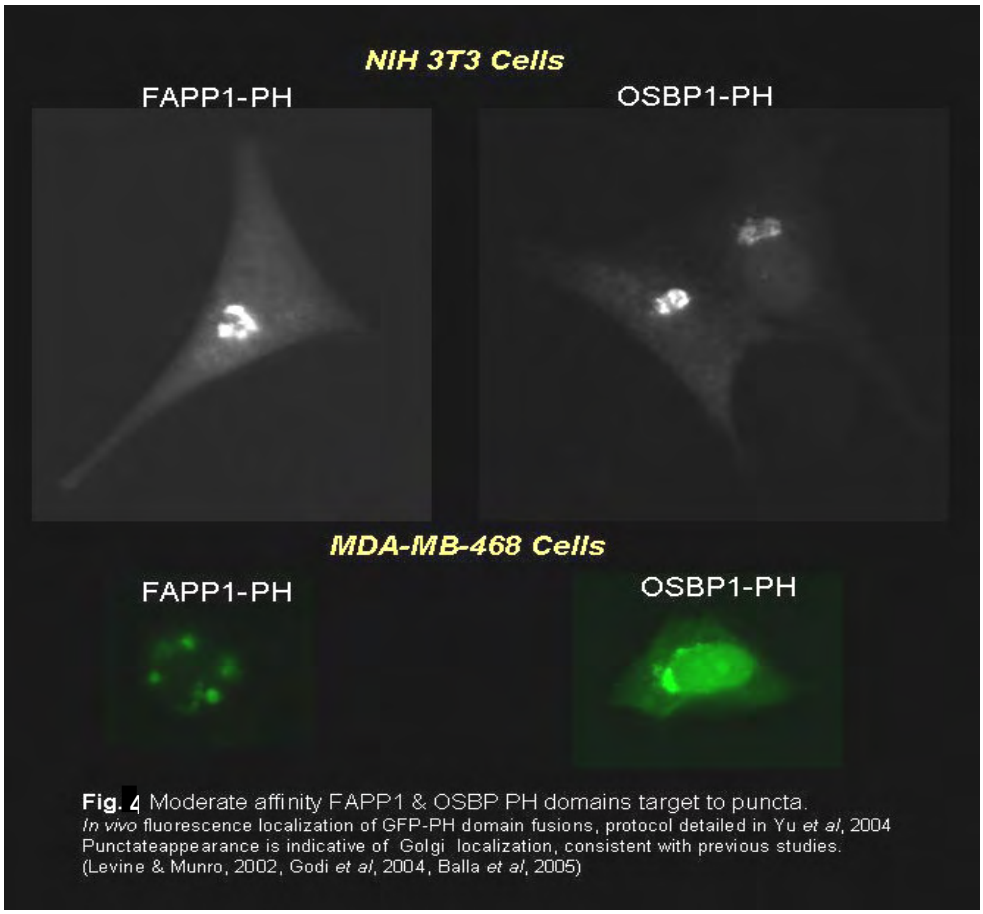


Fig. 4 Moderate affinity FAPP1 & OSBP PH domains target to puncta.
In vivo fluorescence localization of GFP-PH domain fusions, protocol detailed in Yu *et al*, 2004
Punctateappearance is indicative of Golgi localization, consistent with previous studies.
(Levine & Munro, 2002, Godi *et al*, 2004, Balla *et al*, 2005)

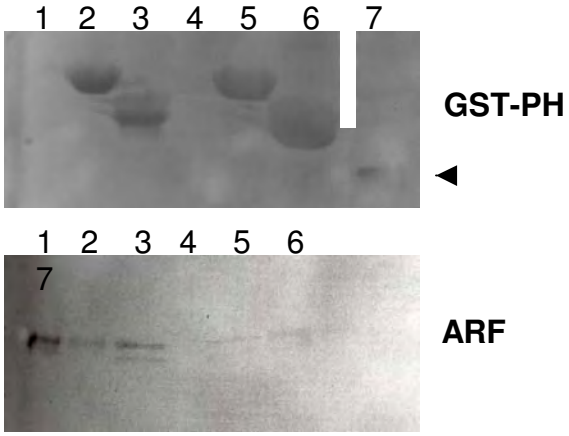


Figure 5: GST pulldown data suggest weak myrArf1 interactions with OSBP and FAPP1 PH.Recombinant myristoylated Arf1 was expressed and purified as previously described (Cesareni *et al*, 2004). GST-OSBP-PH and GST-FAPP1-PH were expressed and purified as previous GST-tagged proteins, with the exception that they were retained immobilized, and not eluted, on the glutathione-Sepharose beads (Lemmon *et al*, 1995; Yu *et al*, 2004). myrArf1 was loaded with 100 μM GTP-γS or GDP by a 1 hr incubation at 32^0C in HEPES loading buffer, followed by a 1 hr. incubation with GST-PH domain immobilized on glutathione-Sepharose beads at RT, as described previously (Godi *et al*, 2004). Beads were collected by low-speed centrifugation, washes 3X, and resuspended in 3X sample buffer. Sample was boiled for 5 min at 95^0C, and run on a 15% SDS-PAGE. Proteins were transferred to nitrocellulose paper by Western blot, blocked for 10 min in Blotto buffer with 5% dry milk, and incubated with **A)** mouse anti-GST antibody (1:1000, QIAgen), or **B)** goat anti-Arf1 antibody (1:1000, Santa Cruz Biotech) in 1X PBS O/N at 4^0C. The blot was washed 3X with 1X PBS, followed by a 1 hr incubation at 4^0C with secondary antibody (rabbit anti-mouse (Amersham) and donkey anti-goat (Santa Cruz Biotech), respectively), and washed again 3X. Finally, the blot was developed with ECL reagents, as per the manufacturer's (Amersham) instructions.

Lanes 1 myrARF1
Lanes 2, 5 myrARF1+GST-OSBP1 PH
Lanes 3, 6 myrARF1+GST-FAPP1 PH
Lanes 7 GST alone

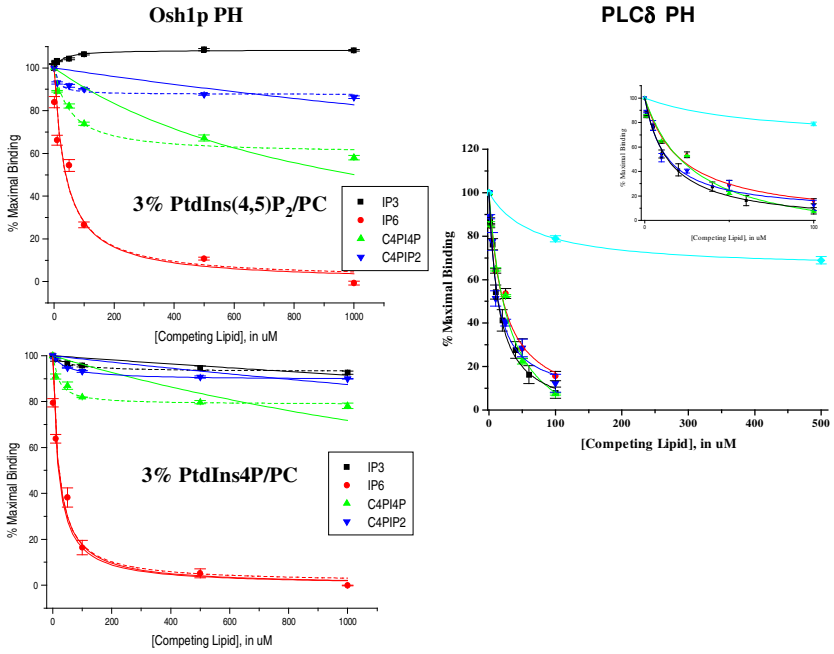


Figure 6: SPR competition studies of Osh1p PH and PLCδ PH on a PtdIns(4,5)P_2 (*top panels*) and Osh1p PH on PtdIns4P (*bottom panel*) surfaces, with indicated InsP/sc PPIns preincubated with the PH domain before flowing over a lipid surface. All datapoints (Rmax) are normalized against non-precomplexed OSBP standard and fit to the curve: $\%_{comp}=\{\%_0*[IC_{50}/(C+IC_{50})]\}+\%_\infty$ where $\%_{comp}$ is RUs at defined concentration, C; $\%_0$ is the Bmax (where inhibition plateaus); IC_{50} is the concentration of competing lipid at 50% inhibition; $\%_\infty$ is the coefficient of variance (CV).

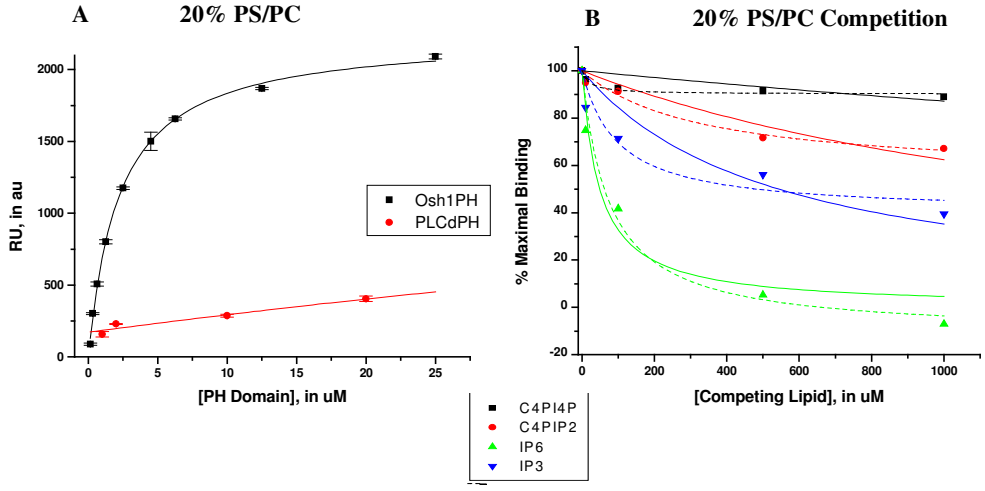


Figure 7: Osh1p PH binds to 20% PtdSer/PC surface (A) and is competed off in a charge-dependent manner (B). Protocols for binding (A) and competition (B) SPR assays as described previously.

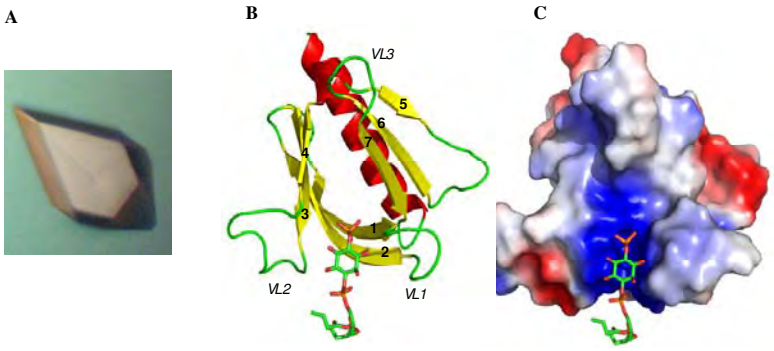


Figure 8: PtdIns4P-Osh1p PH Crystal (A), Ribbon (B) and Electrostatic (C) Structure
His-tagged Osh1p PH domain was crystallized at high concentrations (~10 mg/mL), mixed short chain (C4) PtdIns(4)P or PtdIns(4,5)P_2 in a 1:1.5 ratio in 0.1 M NaAc pH 4.5, 30% PEG-2000MME, 0.2M $(NH_4)_2SO_4$, and incubation at 21ºC for 1 week (A). The crystals were flash-frozen with MPD and examined at a synchrotron source. The data was then processed and refined with HKL2000 and CCP4 suite programs and the structure modelled using COOT program. The ribbon diagram (B) and electrostatic structure (C) above were generated using Pymol.

Summary of Crystallographic Data

	Osh1 PH/PI(4,5)P_2 *"SO4-liganded"*	Osh1 PH/PI4*P* *"PI4P-liganded"*
Data Collection Statistics		
Space group	$P2_12_12_1$	$P2_12_12_1$
Unique cell dimensions (a, b, c)	37.7, 46.3, 63.1 Å	38.7, 46.8, 63.4 Å
X-ray source	X29A	BM23
Resolution limit	1.37 Å	1.5 Å
Observed/unique	92,648 / 20,740	114,871 / 19,062
Completeness (%)	94.0 (86.0)	97.4 (94.6)
Rmerge	0.053 (0.214)	0.098 (0.55)
I/s	32.4 (5.2)	9.8 (2.9)
Redundancy/Multiplicity	4.5	6.0
Molecular Replacement search model	Polyalanine Substitution of DAPP1 PH	
Refinement Statistics		
Resolution limits	32.4-1.37 Å	19.3-1.90 Å
No. of reflections/no. test set	22,189 / 1,435	>19,000 / 606*
R factor (R free)	19 (21)	19 (25)
Vm	1.89 $Å^3$/Da	1.94 $Å^3$/Da
Solvent (%)	34.4	36.0
B value (mean)	22.9 $Å^2$	29.7 $Å^2$
B value (from Wilson Plot)	23.0 $Å^2$	26.0 $Å^2$
Model		
Protein	aa 279-383	aa 279-383
Ligand	None	C4-PtdIns4*P*
Bound ions	3 SO_4	None
Bound water molecules	174	179
RMS deviations		
Bond length	0.008 Å	0.022 Å
Bond angles	1.2 Å	1. 964 Å
Crystallization Conditions		
Mother Liquor	0.1M NaAc pH 4.6 20% PEG-2000ME(w/v) 0.2M $(NH_4)_2SO_4$	0.1M NaAc pH 4.6 20% PEG-2000ME 0.2M $(NH_4)_2SO_4$
Molar Ratio (PH:PPIns)	1:1.5 (1X)	1:1.5 (1X)

* As the structure has not yet been fully refined, the number of reflections is subject to change.

Table 3: Summary of Crystallographic Data

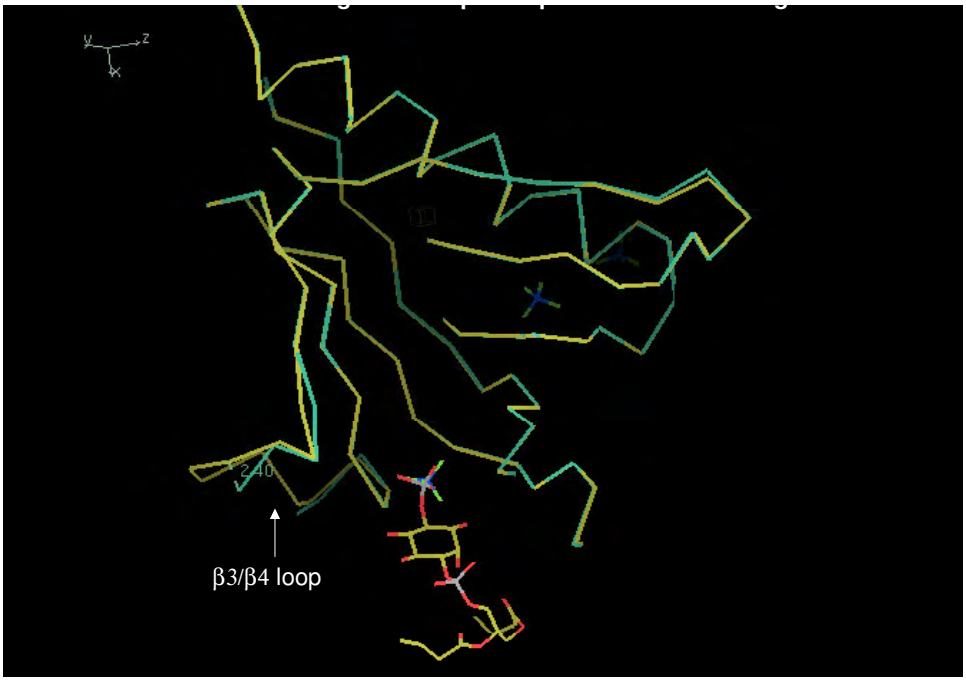


Figure 9: Conformational Change of Osh1p PH Upon PtdIns4P Binding generated by SSM superposing Cα backbone traces in COOT modelling program. SO_4- and PtdIns4P-liganded Osh1p PH traces are in blue and yellow, respectively.

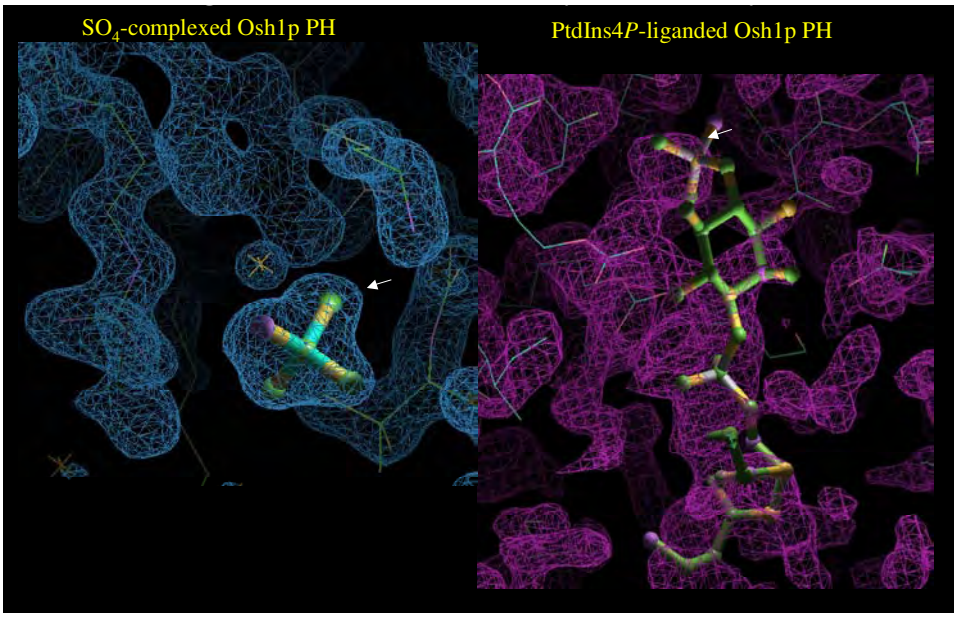


Figure 10: Ligand Coordinated Surrounded by Electron Density in SO_4-(A) and PtdIn4P-liganded (B) Osh1p PH Binding Site generated by script found on COOT website. Free SO_4 and 4-phosphate in similar relative coordinate positions are labeled.

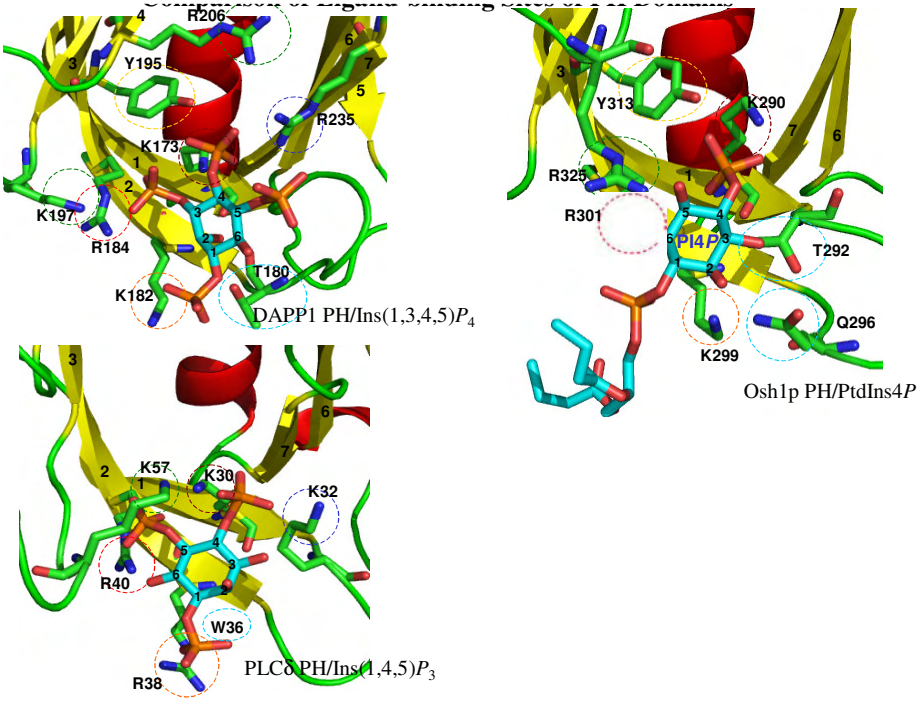


Figure 11: Comparison of Ligand-binding sites of PH domains of DAPP1, Osh1p, and PLCδ generated by Pymol, with key PPIns-interacting side chains labeled.

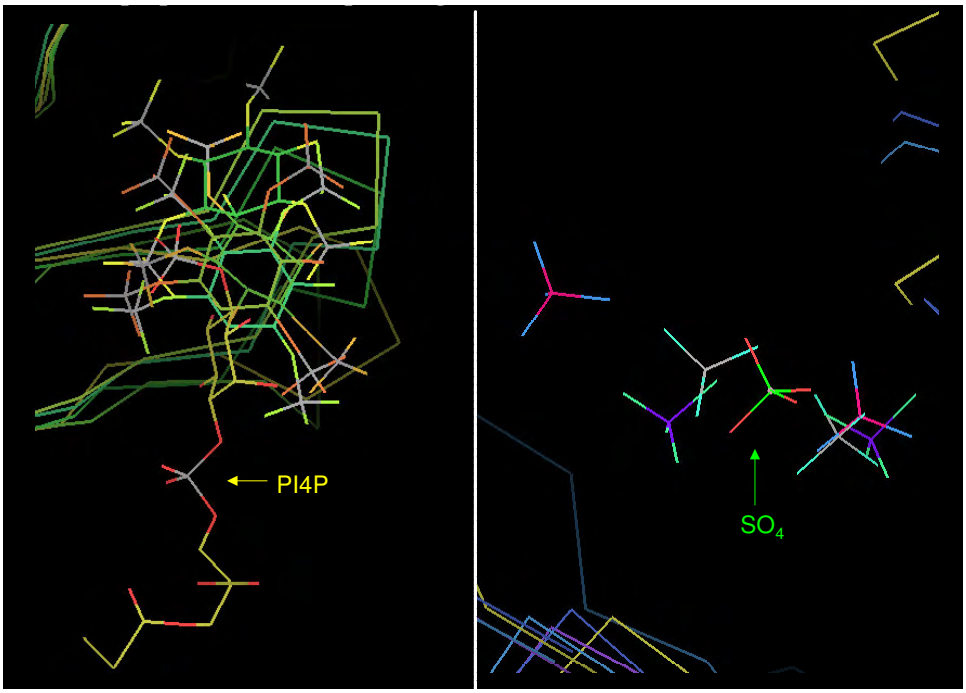


Figure 12: Superposition of Osh1p PH Ligands with those of Other PH Domains generated by SSM function in COOT modeling program. PtdIns4*P* and free SO_4 ion are labeled.

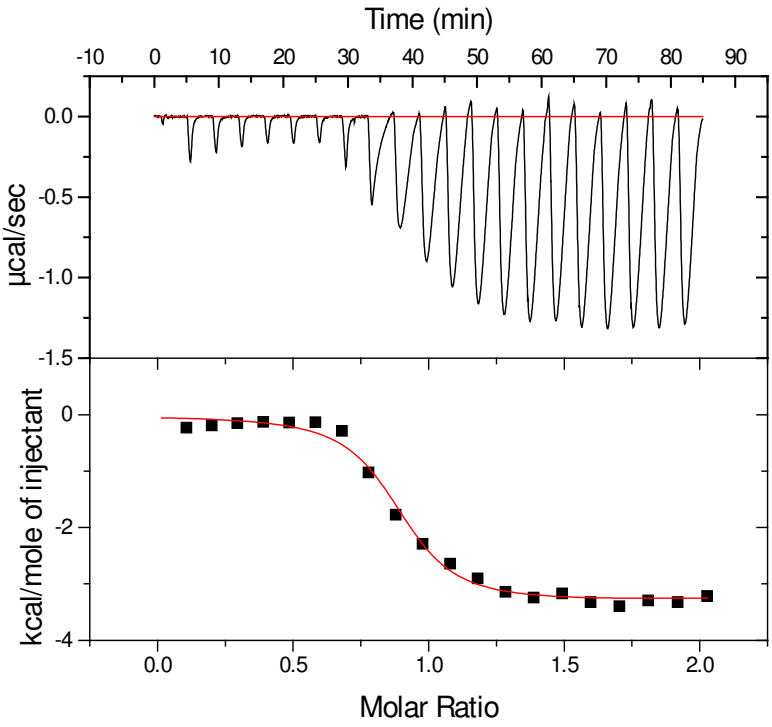


Figure 13: ITC binding data suggest perifosine's weak binding affinity for Akt1/PKBα PH. GST-Akt1/PKBα PH was expressed and purified as previously described (Thomas *et al*, 2002). The protein was dialyzed O/N at 4^0C into 20 mM HEPES pH 7.5, 100mM NaCl, 1 mM DTT and concentrated to 360 µM. The protein was added to the 2 mL ITC cell, while perifosine (3.6 mM) filled the 250 µL syringe. Each injection was 12.5 µL for a total of 20 full injections. The binding curve was fitted with the two-sets of sites model in Origin.